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Genetics

Characterization of genetic variation and 3′-azido-3′-deoxythymidine-resistance mutations of human immunodeficiency virus by the RNase A mismatch cleavage method

(genetic quasi-species/point mutation)

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ABSTRACT **The RNase A mismatch cleavage method has been applied to the characterization of natural genetic variation of human immunodeficiency virus (HIV) from different geographical areas. The approach provides a rapid and simple assay for the analysis of differences in closely related viral isolates and allows the establishment of phylogenetic relationships between epidemiologically distinct viruses. Our results show a broad clustering of circulating viruses according to their geographical distribution. We also have analyzed the temporal appearance of mutations associated with the acquisition of resistance to 3′-azido-3′-deoxythymidine (AZT). The results show that mutations in codon 215 of the viral reverse transcriptase can be detected readily by this method in HIV isolates and also directly in peripheral blood from HIV-infected individuals after *in vitro* amplification of viral sequences with the polymerase chain reaction. The specific recurrence of identical double-nucleotide substitutions in epidemiologically and geographically distant viruses suggests that the restricted amino acid substitutions at this position selected by drug exposure are a critical, rate-limiting step in the acquisition of drug resistance.**

Human immunodeficiency virus (HIV) is a retrovirus of the lentivirus family, which is responsible for AIDS and other related diseases (1, 2). The great genetic variability of HIV (3), a common property of RNA viruses (4, 5), which reflects the quasi-species distribution of the viral populations (6), has profound implications in its biology and pathogenicity (7). Marked variations in antigenic reactivity and nucleotide sequence have been also documented in isolates from different individuals (8). The availability of simple assays to discriminate and characterize viral isolates could help to address many unanswered questions regarding the biology and epidemiology of the virus, such as the appearance of new phenotypic variants, their geographical distribution, and the role of immunity in the selection of variants (9).

Although presently there is no cure for the disease, 3′-azido-3′-deoxythymidine (AZT; also called zidovudine) has prolonged survival and reduced morbidity in patients in several stages of HIV infection (10). Unfortunately, consistent with the intrinsic high mutation rate of RNA viruses (11), resistance to the drug appeared after prolonged therapy (12). The AZT-resistant phenotype is attributable to four amino acid substitutions in the viral reverse transcriptase (RT), which are the result of single nucleotide changes in codons 67, 70, and 219 and of two adjacent base substitutions in codon 215 (13).

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The ability of RNase A to recognize and cleave single-base mismatches in RNA·RNA and RNA·DNA hybrids can be exploited for the detection of point mutations (14, 15) and for the analysis of genetic variability of RNA viruses (16, 17). We have used this approach for the characterization of genetic variation of HIV isolates of different epidemiological origin and geographical distribution and for the detection of viruses resistant to AZT.

MATERIALS AND METHODS

Origin and Isolation of Viral Strains. The viruses used in this study (Table 1) were from patients with AIDS or AIDS-related complex (ARC), mainly homosexuals in San Diego, CA, or intravenous (i.v.) drug abusers and infected children of i.v. drug abusers in Madrid, Spain. A set of viruses from five individuals on AZT therapy (samples 12, 18, 22, 26, and 36) has been described (12, 13) and includes samples obtained at the beginning of the treatment (i.e., 12/1) and subsequent isolates (i.e., 12/2). HIV isolates originally called human T-cell lymphotropic virus type III—namely, HTLV-IIIB (ref. 18; henceforth referred to as IIIB) and HTLV-IIIRF (ref. 19; henceforth referred to as RF)—and HIV isolate SF-2 (ref. 20; originally called ARV-2) were used as reference strains. Viruses were isolated by cocultivation of peripheral blood lymphocytes (PBL) from infected individuals with PBL from healthy donors. When positive for p24 antigen (Abbott), cultures were grown in H9 or MT-2 human T cells for one or two passages as described (21). For the AZT studies, viruses recovered by transfecting MT-2 cells with clones HXB2-D (a clone of IIIB isolate) and pHIVRTMC were used as negative and positive controls, respectively. pHIVRTMC, a gift from B. Larder, is the HXB2-D clone with the four mutations at codons 67, 70, 215, and 219 in the RT gene that confer the resistant phenotype to AZT introduced by *in vitro* mutagenesis (13).

Plasmids. Plasmid pBH10-R3 (gift of R. Gallo) is an almost full-length clone of the HIV IIIB isolate genome (18) cloned into the *Sst* I site of pSP64 (Promega). Plasmid pEH was constructed by deleting the 4.2-kilobase (kb) 5′-end *Eco*RI fragment of pBH10-R3 by partial digestion with this enzyme. Subsequently, the 1.5-kb 3′-end *Hin*dIII fragment was also deleted. pPSP is the 1.2-kb *Pvu* II–*Spe* I fragment of pP-1 (at positions 2878 and in the polylinker, respectively) cloned into the pBluescript SK(−) vector (Stratagene). pP-1 is the 2.5-kb

Abbreviations: ARC, AIDS-related complex; HIV, human immunodeficiency virus; AZT, 3′-azido-3′-deoxythymidine; RT, reverse transcriptase; HTLV-III, HIV isolates called human T-cell lymphotropic virus type III; PBL, peripheral blood lymphocytes; PCR, polymerase chain reaction; nt, nucleotide(s).

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Table 1. Detection of AZT-resistance mutations in HIV by the RNase A mismatch method

Case	Months*	Sensitivity†	RNase‡	Sequence§
		San Diego, CA		
12/2	1	0.01	−	WT
12/4	25	2.0	+	MUT
18/1	0	0.01	−	WT
18/3	16	4.0	+	MUT
22/1	0	0.03	−	WT
22/3	15	5.6	+	MUT
26/1	0	0.01	−	WT
26/2	11	2.8	+	MUT
36/2	2	0.01	−	WT
36/4	17	0.67	(+)	MUT
36/5	25	5.6	(+)	MUT
21-A	0	ND	−	ND
24-A	0	ND	−	ND
2-B/1	0	0.01	(+)	WT
2-B/2	4	0.05	+	WT/MUT
4-B	4	0.01	−	WT
5-B	16	0.06	+	MUT
7-B/1	0	0.01	−	WT
7-B/2	16	0.18	(+)	MUT
8-B	12	0.14	(+)	WT
9-B	13	0.09	+	MUT
10-B	14	0.09	(+)	ND
11-B	12	0.17	+	MUT
		Madrid, Spain		
58	0	ND	−	ND
59	0	ND	−	ND
61	0	ND	−	ND
75	0	ND	−	ND
80	0	ND	−	ND
106	0	ND	−	ND
108	11	ND	+	MUT
923	0	ND	−	ND
1067	14	ND	+	MUT
1578/1	0	ND	−	ND
1578/2	0	ND	−	ND
1862	<6	ND	−	WT

HIV isolates were from San Diego or Madrid patients, all of which were males except 59, 61, 75, 106, 108, and 1578. Samples 1578/1 and 1578/2 were taken 13 months apart.

*Months on continued AZT therapy.

†Values indicate concentration of AZT (μM) for 50% inhibition in the *in vitro* sensitivity assay (12). IC_{50} values of <0.05 are considered sensitive (wild type) in phenotype.

‡Presence (+) or absence (−) of the 214-nucleotide (nt) and 109-nt bands diagnostic of mutation in codon 215 in the RT gene; "(+)" indicates an incomplete pattern (one or more bands were missing; see text).

§Sequence of codon 215 determined after amplification by the polymerase chain reaction (PCR): Wild type (WT), ACC (Thr); mutant (MUT), TTC (Phe, isolates 12, 22, and 108) or TAC (Tyr, isolates 18, 26, and 36). Isolate 1067 was a mixture of both mutations. ND, not determined.

Bgl II–*Eco*RI fragment of pBH10-R3 (at positions 1640–4228) that spans most of the polymerase (*pol*) gene, cloned into the pBluescript SK(−) vector. The 520-nt ENV riboprobe was synthesized with the phage SP6 RNA polymerase after linearizing pEH with *Bgl* II. The 323-nt RT 3′ riboprobe was generated with the phage T7 RNA polymerase after linearizing pPSP with *Eco*RV.

Hybridizations and RNase Digestions. Uniformly labeled RNA probes were synthesized as described (16) by using [^{32}P]CTP (Amersham); 5×10^5 to 10^6 cpm of probe was hybridized to $\approx$1 μg of total cellular RNA for 4 hr at 55°C. Total RNA was obtained from *in vitro* infected H9 or MT-2 cells or PBL as described (14). Hybrids were digested with bovine pancreatic RNase A (40 μg/ml; Pharmacia) at 30°C for 30 min. For the experiments with PCR-amplified DNA, the RNA probe was synthesized in the presence of 200 μM CTP; 10^5 cpm of probe was hybridized to 1 μl of the PCR product at 45°C for 7 hr. Digestions were at 30°C for 15 min. The RNase A resistant fragments were analyzed by denaturing polyacrylamide gels as described (16).

PCR. Amplification with a thermostable *thermus aquaticus* (*Taq*) DNA polymerase (Cetus) was performed as described (22) in a Perkin–Elmer Thermocycler with a profile of a denaturing step at 94°C for 35 sec, an annealing step for 1 min at 50°C, and an elongation step for 1 min at 72°C. PCR primers (Genetic Designs, Houston) 1U (5′-GGGATTAGATATCAGTACAATGTGCTT-3′) and 1D (5′-CGCTCGAGTTCATAACCCATCCAAAGG-3′) correspond to positions 2550–2577 and 2810–2836 of HIV clone BH10 (18), respectively. Nested primers 2U (5′-GGATCACCAGCAATATTCCAAAGT-3′) and 2D (5′-GCGCTCGAGGTTCTTTCTGAT-3′) correspond to positions 2592–2616 and 2791–2811, respectively. To analyze HIV sequences directly from PBL, an initial reverse transcription reaction was performed as described (23) by using total RNA and a primer (5′-TCAGTCCAGCTGTCTTTTTCTGGC-3′) corresponding to positions 2867–2890. Ten microliters of the reverse transcription reaction was used for a first round of PCR with primers 1U and 1D for 25 cycles. One microliter of the reaction was used in a second PCR with primers 2U and 2D for 33 cycles.

RESULTS

Molecular Epidemiology of HIV. The RNase A mismatch cleavage method is based on the ability of RNase A to recognize and cleave single-base mismatches in RNA·RNA or RNA·DNA hybrids. The marked sequence differences in the genome of RNA viruses generate heterohybrids with many mismatches, which after RNase treatment generate electrophoretic band patterns that are characteristic fingerprints for each strain. Comparative analysis of different viral isolates can readily reveal genetic variations between closely related viruses. At the same time, it can provide an estimation of the degree of relatedness between different isolates by the presence, number, and relative size of common bands because they represent similar, if not identical, nucleotide sequence differences relative to the strain used as probe.

To study the natural variation of HIV, we analyzed viruses isolated from the same and different patients and from different geographical locations. The riboprobes used in this study are shown in Fig. 1 *Top*. The ENV probe spans the RNA encoding the N-terminal region of the gp40 protein and a small fragment of the C terminus of the gp120 product. This region shows a 6–7% variation between reference strains (24). Two other more conserved regions are covered by the RT and GAG probes with 3–4% interstrain variation (24). The results for the ENV and RT 3′ riboprobes are shown in Fig. 1. These experiments show the high degree of genetic heterogeneity of HIV as reflected by the distinct patterns of protected bands obtained for the different isolates. Temporal genetic variability within the same patients (1578 and 7-B), was also apparent, in both *env* and the RT gene.

Despite this marked diversity, similarities in the *env* gene were also evidenced among different isolates by the presence of subsets of common bands. For instance, Spanish isolates 61, 1578, and 1910 or Californian isolates 11-B, 9-B, 5-B, and 7-B/2 shared two or more bands, but no common band was present between these Spanish and Californian viruses. Many isolates from San Diego also appeared to be related to the Californian reference strain SF-2 as evidenced by the presence of common bands marked by asterisks at the right. Some of these viruses exhibited more than three common bands (11-B, 8-B, 5-B, 7-B/2, and 26/1), and others exhibited

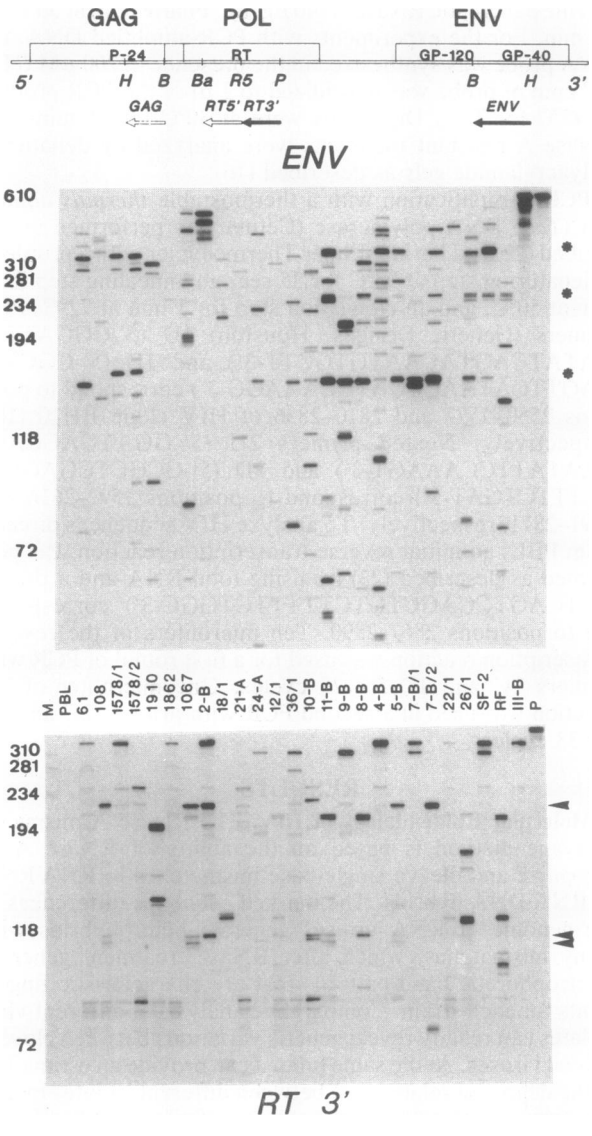

FIG. 1. RNase A mismatch cleavage analysis of RNA·RNA heteroduplexes between different HIV isolates and antisense riboprobes of the IIIB reference strain. (*Top*) Map of the HIV genome and the riboprobes used (GAG; RT 5'; RT 3'; and ENV). The restriction endonucleases delimiting the riboprobes are: H, *Hin*dIII; B, *Bgl* II; Ba, *Bal* I; R5, *Eco*RV; and P, *Pvu* II. The solid arrows indicate the riboprobes for which experiments are shown below. (*Middle* and *Bottom*) Total cellular RNA was obtained from cultured cells infected with the virus isolates indicated vertically between the gels. The RNA hybrids were digested with RNase A, and the resistant fragments were electrophoresed in urea/polyacrylamide gels. The gels were exposed to x-ray films for 1–3 days at −70°C. Lanes: M, ϕX174 digested with *Hae* III to give molecular weight markers; PBL, total RNA of uninfected control PBL; 61 through 1067, seven isolates from Madrid; 2-B through 26/1, 16 isolates from San Diego; SF-2, RF, and IIIB, reference isolates; P, undigested probes (400 cpm). The numbers indicate the position and size in nucleotides of the fragments. Asterisks at the right of *Middle* highlight the bands that are characteristic of the viruses related to the SF-2 reference strain. Arrowheads at the right of *Bottom* indicate the bands diagnostic of resistance to AZT.

at least two (36/1, 10-B, 9-B, 4-B, and 7-B/1) common bands. Another group of viruses (2-B, 18/1, 21/1, and 22/1) yielded fewer and larger bands, suggesting a closer relation to the IIIB/LAV reference isolate of HIV. The absence of bands characteristic of the SF-2 reference strain was shared by the Spanish isolates with the exception of virus 61.

Detection of HIV Mutants Resistant to AZT. When the region of the viral genome corresponding to the area encoding RT was similarly analyzed (Fig. 1 *Bottom*), several common bands were also observed in various isolates (for instance, isolates 36/1, 9-B, and 7-B/1). Analysis of the clinical data revealed a possible origin for other common bands, indicated by arrows at the right. All viruses yielding at least two of these bands (108, 1607, 2-B, 9-B, 11-B, and 5-B), with the exception of 2-B, were isolated from patients on AZT therapy (see Table 1). These bands could be due to the mutation(s) responsible for the acquisition of the resistant phenotype to the drug (13). Two of the four described mutated positions (codons 215 and 219) were covered by the RT 3' probe.

The origin of these bands was confirmed in another experiment (Fig. 2). While the wild-type HXB2-D virus generated the expected 323-nt protected RNA band (Fig. 2 *Upper*), clone HIVRTMC to which the mutations responsible for the resistance to AZT were introduced by *in vitro* mutagenesis (13) generated a more complex pattern of bands, including the 214-, 109-, and 100-nt bands diagnostic of mutations at codons 215 and 219 (Fig. 2 *Lower*). Other bands also present are due to the existence of nucleotide changes in the clone pBH10-R3 used for synthesis of the probe (see below),

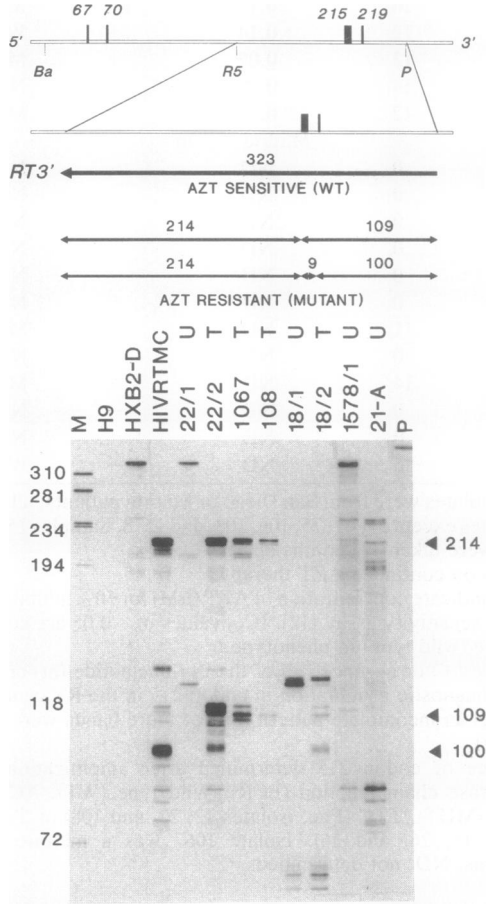

FIG. 2. Identification of AZT-resistant HIV genotypes by the RNase A mismatch method. (*Upper*) Empty lines represent the polymerase gene region. Solid vertical bars signal the localization of the mutations responsible for the AZT-resistant phenotype, with the italic numbers indicating the corresponding amino acids in the RT. The region from *Pvu* II (P) to *Eco*RV (R5) coinciding with the RT 3' probe used in the gel displayed in *Lower* is shown with the sizes in nucleotides of the expected RNase A-protected RNA fragments of wild-type (AZT sensitive) and mutant (AZT resistant) viruses, respectively. (*Lower*) Lanes: M and P are as in Fig. 1; H9, total RNA from uninfected H9 cells; HXB2-D and HIVRTMC, RNAs from cultured cells infected with these clones, which were used as controls for wild-type and mutant viruses, respectively; 22/1 through 21-A, RNAs from cultures infected by isolates as indicated.

degradation of the RNA, and/or the quasi-species nature of the viral populations.

Two pairs of isolates (22 and 18) obtained either before (Fig. 2 *Lower*, lanes U) or after (lanes T) prolonged AZT treatment, previously characterized for their resistance to AZT *in vitro* and by nucleotide sequence (12, 13), were also analyzed. Two uncharacterized viruses from AZT-treated Spanish patients (1067 and 108) and two from untreated individuals (1910 and 1578) were also included. As with the HIVRTMC-positive control, the bands diagnostic of mutations at codons 219 and/or 215 (indicated with arrowheads at the right) were present in viruses 22 and 18 after AZT treatment but were absent before AZT treatment and in the untreated isolates. Because Spanish isolates 1067 and 108 also generated the 214- and 109-nt bands, this result suggested that they were already mutant, although only at codon 215.

To confirm or rule out this interpretation, we amplified the genomes from isolates 1067 and 108 by the PCR and sequenced the PCR products (data not shown). Isolate 108 had TAC (tyrosine) at codon 215, while isolate 1067 showed a mixed population with TAC (tyrosine) and TTC (phenylalanine). These are precisely the same mutations previously described for other AZT-resistant strains (13). As predicted by the RNase A mismatch experiment, both viruses lacked the mutation at codon 219.

The results of our analysis of a panel of different HIV isolates are summarized in Table 1. With the exception of isolate 2-B, the presence of the 214- and 109-nt bands (see also Fig. 1) correlated with prolonged AZT therapy, with resistance to AZT *in vitro*, and with a mutated codon 215.

Detection of HIV AZT-Resistant Mutants in PBL. To circumvent the need of virus isolation by cocultivation, we used a combination of *in vitro* amplification by the PCR, followed by mismatch analysis of the amplified DNA·RNA hybrids to identify directly the mutations in viruses present in the blood of infected individuals. The results of a mismatch analysis of PCR-amplified HIV DNA sequences using total cellular RNA from PBL are shown in Fig. 3. When PCR-amplified DNA from cultured cells infected with virus HXB2-D (Fig. 3 *Lower*, lane 1) was subjected to RNase A mismatch analysis, in addition to the 219-nt band corresponding to the protected RNA·DNA hybrid, other bands of 166 and 53 nt were generated. These RNA fragments correspond to substantial RNase cleavage at the mismatch present at codon 172 (see Fig. 3 *Upper*) due to a nucleotide change in the clone of the IIIB HIV isolate pBH10-R3, used for synthesis of the riboprobe. The presence of one or few nucleotide changes in an individual HIV clone is not surprising because of the quasi-species nature of RNA viruses (6, 11, 25).

When DNA amplified from HIVRTMC provirus (Fig. 3 *Lower*, lane 2) was similarly processed, it generated the expected bands of 180 and 127 nt specific for RNase A cleavage at the mismatch in codon 215. Viruses 11-B and 5-B (lanes 3 and 4) also generated the bands diagnostic of the presence of a mutation at codon 215 of the RT gene, in agreement with the results of Fig. 1. These results indicate that the circulating viral population from patients 11.B and 5.B contained the double mutation in codon 215 and demonstrate the feasibility of this approach to detect AZT-resistance mutations directly from peripheral blood of HIV-infected individuals.

DISCUSSION

We have shown that the RNase A mismatch cleavage method can be used for the characterization of genetic heterogeneity and variability of HIV field isolates. The high resolution achieved in these experiments by standard sequencing gels not only increases the sensitivity to detect differences between closely related viruses but also provides a level of accuracy sufficient to establish meaningful relationships between different viral isolates. In these gels, the level of resolution of a single band after autoradiography is about 2 mm, or $\approx 10^{-2}$ of the length of the relevant region of the gel. This value can be considered to be the probability for two RNA fragments to comigrate by coincidence, which therefore is not sufficient for determining an interstrain genetic relatedness. However, the probabilities of coincidental identical mobilities for two and three bands will be 10^{-4} and 10^{-6}, respectively, which provides a reasonable level of confidence for establishing sequence similarities and phylogenetic relationships between different viral genomes. The application of this technique may be particularly relevant for the establishment of infectious identity between viruses (see Fig. 1, isolate 1578) in certain situations, as between family members or patients and health care providers. The exclusion of identity on the other hand is not possible because only a few nucleotide changes might disrupt the similarities of the RNase mismatch patterns (see Fig. 1, isolate 7-B).

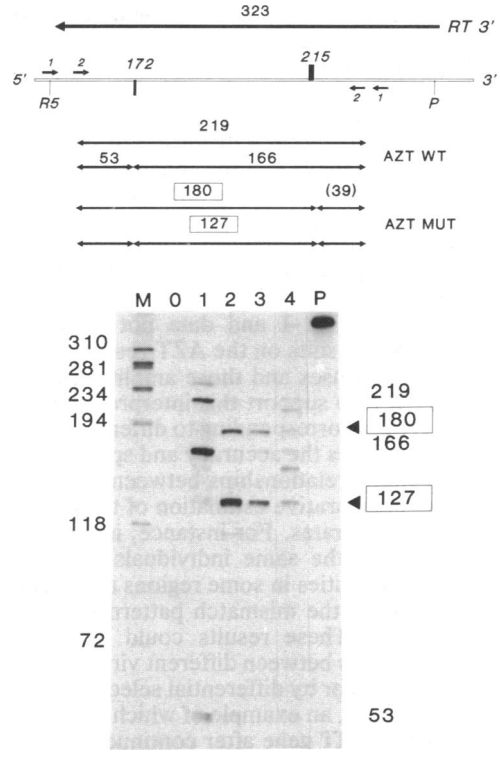

FIG. 3. Detection of AZT-resistance mutations in HIV sequences amplified by the PCR from lymphocytes of infected individuals. (*Upper*) Sizes of the expected RNA fragments by RNase A cleavage of the RNA·DNA hybrids after two consecutive PCR reactions, the second with nested primers (small arrows). Solid vertical bar with number 172 on top indicates a mutation present in the clone pBH10-R3 used for synthesis of the riboprobe. The PCR products and sizes of the expected RNase A-protected RNA fragments of the AZT wild type (WT; AZT sensitive) and AZT mutant (MUT; AZT resistant) viruses are shown with square boxes highlighting the bands diagnostic of AZT resistance. (*Lower*) RNase A mismatch assay of PCR-amplified DNA using total genomic DNA from cultured cells infected with clones HXB2-D and HIVRTM (lanes 1 and 2) or total PBL RNA from cases 11-B and 5-B (lanes 3 and 4). Lanes: M and P, as in Fig. 1; 0, no RNA added to the PCR.

We have detected genetic relatedness between several viruses circulating in the population of homosexuals in San Diego that also show similarities to the Californian SF-2 reference strain. The relation of San Diego HIV isolates to the SF-2 prototype, which was not found in the circulating Spanish viral population, together with the absence of common bands between these two groups of viruses, suggest different origins for the majority of the viruses from these

geographically distinct areas. At the same time, the presence of detectable similarities between isolates within these areas suggests a significant epidemiological clustering of viruses according to their geographical localization.

Although we have used in our studies viruses grown in culture, which may result in a distortion of the viral master sequences (25), we believe that the conclusions regarding the phylogenetic relationships among different isolates are valid because they are derived by relative comparative analysis of their corresponding RNase mismatch patterns. The identical or very similar mismatch patterns frequently obtained with viruses isolated simultaneously or after short intervals from the same patients (Fig. 1 and data not shown) and the concordance of the results on the AZT-resistance mutations between cultured viruses and those amplified directly from PBL by the PCR also support this interpretation.

The use of probes corresponding to different regions of the viral genome increases the accuracy and specificity in establishing phylogenetic relationships between viruses and also allows an easy comparative estimation of their relative variability and mutation rates. For instance, in viruses sequentially isolated from the same individuals, we have found striking genetic stabilities in some regions of the genome but dramatic changes in the mismatch pattern in other regions (data not shown). These results could be explained by recombination events between different viruses coexisting in the same individuals or by differential selective pressures for fixation of mutations, an example of which is the occurrence of mutations in the RT gene after continued AZT therapy.

We have taken advantage of the ability of the RNase A mismatch cleavage method to detect point mutations (14–16) to identify the temporal appearance of resistance to AZT in HIV isolates from patients with AIDS or ARC receiving prolonged therapy. The reported occurrence of a double nucleotide change at codon 215 in AZT-resistant viruses (13) was especially helpful because double mismatches are efficiently cleaved by the enzyme. This double mutation is apparently due to the requirement for restricted amino acid changes, from the threonine present in the wild-type sequence (ACC) to a tyrosine (TAC) or to phenylalanine (TTC). By showing that identical double mutations occurred in epidemiologically and geographically distant viruses, our studies have confirmed the specific association of the double nucleotide change at codon 215 with the acquisition of the AZT-resistant phenotype. Our screening of HIV isolates failed also to detect circulating viruses with the mutated genotype in the absence of prolonged AZT therapy (Table 1). Whether this is simply due to the small or early sample of isolates studied or to a less viable viral phenotype provided by the AZT mutations remains to be seen. In this context, our method could be useful also because not only would it detect the presence of the mutations, but also it could establish the epidemiological interrelationships of the corresponding viruses.

Detection of the mismatch-specific bands corresponding to mutations at codon 215 of the viral RT gene by the RNase A method should have applications to determine the time of acquisition of the AZT-resistant phenotype. This may prove to be useful to decide on the choice of further therapy. Acquired resistance to other antiviral drugs for HIV and other viruses (10), resulting from the acquisition and subsequent selection of mutations leading to single amino acid substitutions, could be similarly analyzed by the experimental approach described in this work.

Recently, detection of RT codon 215 mutations has been reported in AZT-treated HIV isolates by PCR and oligonucleotide hybridization (26).

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